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Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits

JOHN F. KEANEY, JR.*, J. MICHAEL GAZIANO*, AIMING XU*, BALZ FREI†, JOANNE CURRAN-CELENTANO‡, GLENN T. SHWAERY‡, JOSEPH LOSCALZO*, AND JOSEPH A. VITA*§

*Department of Medicine, Brigham and Women's Hospital and Brockton/West Roxbury Veterans Administration Medical Center, Harvard Medical School, Boston, MA 02115; †Department of Nutrition, and Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115; and ‡Department of Animal and Nutritional Sciences, University of New Hampshire, Durham, NH 03824

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ABSTRACT **Recent evidence suggests that dietary therapy with lipid-soluble antioxidants may be beneficial for patients with atherosclerotic vascular disease but the potential mechanism(s) for these observations remain obscure. Abnormalities in endothelium-dependent control of vascular tone develop early in the course of atherosclerosis and may result from oxidative modification of low density lipoproteins. We examined the role of dietary antioxidants in preserving normal endothelial cell vasodilator function in cholesterol-fed rabbits with particular attention to possible effects on serum lipoproteins, low density lipoprotein oxidation, and atherogenesis. Male New Zealand White rabbits were fed diets containing no additive (controls), 1% cholesterol (cholesterol group), or 1% cholesterol chow supplemented with either β-carotene (0.6 g/kg of chow) or α-tocopherol (1000 international units/kg of chow) for a 28-day period. After dietary therapy, thoracic aortae were harvested for assay of vascular function and for pathologic examination and tissue antioxidant levels. Compared to controls, acetylcholine- and A23187-mediated endothelium-dependent relaxations were significantly impaired in vessels from the cholesterol group ($P < 0.001$), whereas vessels from animals treated with β-carotene or α-tocopherol demonstrated normal endothelium-dependent arterial relaxation. Preservation of endothelial function was associated with vascular incorporation of α-tocopherol and β-carotene but was unrelated to plasma lipoprotein levels, smooth muscle cell function, or the extent of atherosclerosis. Increased low density lipoprotein resistance to *ex vivo* copper-mediated oxidation was observed only in the α-tocopherol group. Our results suggest that dietary antioxidants may benefit patients with atherosclerosis by preserving endothelial vasodilator function through a mechanism related to vascular tissue antioxidant content and not reflected by assay of low density lipoprotein resistance to *ex vivo* oxidation.**

The vascular endothelium is important in a number of homeostatic functions including the regulation of blood flow, vascular tone, and local platelet function (1, 2). Abnormalities of endothelium-derived relaxing factor (EDRF) action have been described in atherosclerosis (3) and hypercholesterolemia (4) and may contribute to the development of acute vascular syndromes. The oxidative modification of low density lipoprotein (LDL) has been implicated in both atherogenesis and the development of abnormal endothelium-dependent control of vascular tone (5, 6). Dietary antioxidants protect LDL against oxidation (7, 8) and limit experimental atherosclerosis (9), and epidemiologic evidence suggests that dietary antioxidants may prevent the clinical manifestations of coronary artery disease (10, 11).

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Abnormalities in endothelium-dependent arterial relaxation develop early in the course of atherosclerosis (4) and may, in part, result from the effects of oxidized LDL (ox-LDL) on agonist-mediated EDRF release (6, 12) and EDRF degradation (13). *In vitro*, ox-LDL inhibits receptor-mediated endothelium-dependent arterial relaxation (6, 12) and endothelial-cell signal transduction (14). Moreover, ox-LDL is cytotoxic to endothelial cells (15) and chemotactic for monocytes (16) leading to the accumulation of vascular inflammatory cells, the local production of oxygen-derived free radicals, and the degradation of EDRF (17, 18).

Lipoproteins and extracellular fluids contain a number of antioxidant defense mechanisms. The main water-soluble antioxidant in plasma is ascorbate (19), while urate, bilirubin, and protein sulfhydryl species provide less-efficient antioxidant protection (20). Important lipid-soluble antioxidants include α-tocopherol (7, 21), lycopene (7), β-carotene (7), and ubiquinol (22). Oxidation of LDL is limited by the administration of antioxidants. Incubation of LDL with α-tocopherol inhibits copper- and endothelial-cell-mediated modification of LDL (23), and in patients, dietary supplementation with α-tocopherol produces an enrichment of LDL α-tocopherol content and protects the LDL particle from copper-mediated oxidation *ex vivo* (24). Similar findings have been reported with the endogenous antioxidant ubiquinol (22, 25), while the data for β-carotene supplementation are controversial (8, 26).

Despite evidence linking ox-LDL to atherosclerosis and abnormalities in EDRF action, the potential role of antioxidants in attenuating abnormalities in endothelial function remains unknown. We sought to examine the effect of dietary antioxidants on endothelium-dependent control of vascular tone in hypercholesterolemia. We report here the effects of dietary antioxidants on endothelium-dependent vasodilation in cholesterol-fed rabbits as well as effects on serum lipoproteins, LDL oxidation, and atherogenesis.

MATERIALS AND METHODS

Materials. Sodium pentobarbital was obtained from Anthony Products (Arcadia, CA). Porcine heparin was purchased from Elkins-Sinn (Cherry Hill, NJ), and sodium nitroprusside was obtained from Abbott. Glutaraldehyde, formaldehyde, osmium tetroxide, and cacodylate were purchased from Polyscience. Chelex 100 resin (100–200 mesh) was obtained from Bio-Rad and all other compounds were purchased from Sigma. Phosphate-buffered saline (PBS) con-

Abbreviations: LDL, low density lipoprotein; ox-LDL, oxidized LDL; HDL, high density lipoprotein; VLDL, very low density lipoprotein; C, cholesterol; EDRF, endothelium-derived relaxing factor; NS, not significant.

§To whom reprint requests should be addressed at: Department of Medicine, Cardiovascular Division, Brockton/West Roxbury Veterans Administration Medical Center, 1400 VFW Parkway, West Roxbury, MA 02132.

sisted of 10 mM sodium phosphate and 0.15 M NaCl (pH 7.4). Reagents used for LDL experiments were prepared with Chelex-treated double-distilled deionized water to prevent premature LDL oxidation by metal ions. A23187 was prepared and diluted in dimethyl sulfoxide; all other reagents were prepared with distilled water.

Animal Subjects. Forty-eight male New Zealand White rabbits (2.4–3.6 kg) were exposed to dietary treatment for a period of 28 days. Twelve of these animals were fed standard Purina rabbit chow (without vitamin mixture) and served as the control group. Thirty-six animals were fed a diet containing 1% cholesterol with the following supplements ($n = 12$ per group): (*i*) no additive (cholesterol group); (*ii*) β-carotene at 0.6 g/kg of chow (β-carotene group); (*iii*) α-tocopherol acetate at 1000 international units/kg of chow (α-tocopherol group). All diets were prepared by a commercial vendor (Research Diets, Brunswick, NJ) and animals were fed chow at 110 g/day and allowed water ad libitum. Blood was obtained in Vacutainer tubes (4.5 mg of Na_2EDTA per 3 ml) prior to dietary treatment and at the time of sacrifice. Plasma was prepared by centrifugation (1000 × g) for 11 min at 4°C and stored at −70°C (protected from light) for subsequent assay of plasma lipoproteins, carotenoids, and tocopherol species. Plasma was analyzed for the content of total cholesterol, triacylglycerol, and high density lipoprotein-cholesterol (HDL-C), using commercially available kits (Sclavo, Wayne, NJ). Plasma LDL- plus very low density lipoprotein (VLDL)-cholesterol (LDL-C + VLDL-C) was derived from the above data using the modified Friedewald formula VLDL-C + LDL-C = total cholesterol − (HDL-C + 0.16 × triacylglycerol).

***In Vitro* Assay of Vascular Function.** Rabbit aortic rings (3 mm) were suspended in an organ chamber with oxygenated Krebs buffer at 37°C and contracted with 1 μM norepinephrine for the assay of vascular function as described (27). Acetylcholine, A23187, and sodium nitroprusside were added sequentially, and vessel relaxation was reported as the percent reduction in isometric tension from the tension produced by 1 μM norepinephrine. At the end of selected experiments, a representative ring was fixed in 10% (vol/vol) formaldehyde and subjected to scanning electron microscopy to confirm the presence of endothelium. In some experiments, the vascular endothelium was removed by gently rubbing with a moistened cotton swab.

LDL Oxidation Studies. The isolation and oxidation of LDL were performed as described by Retsky *et al.* (28). Briefly, blood (10 ml) was collected from fasting rabbits into sodium heparin in Vacutainer tubes (sodium heparin, 286 USP units/15 ml of blood) and plasma was obtained as described above. Residual ascorbic acid and urate were removed using gel filtration as described (28). LDL was prepared from gel-filtered plasma by the methods of Chung *et al.* (29) using a Beckman NTV90 rotor (443,000 × g_{av} for 45 min) and a Beckman L8-80M ultracentrifuge. LDL protein content was determined by the method of Lowry (30) and isolated LDL was used immediately for experiments. Standard incubation of LDL for susceptibility to oxidation was performed as described (28) and LDL susceptibility to lipid peroxidation was quantified by the lag-phase duration before the formation of conjugated dienes as described by Esterbauer *et al.* (7).

LDL Antioxidant Content. A 200-μl sample containing 0.1 mg of LDL protein in PBS was extracted with 200 μl of methanol and 2.5 ml of hexane. The hexane phase (2.0 ml) was dried under nitrogen, resuspended in ethanol, and analyzed by reverse-phase HPLC using an LC-8 column (Supelco) and 1% water in methanol containing 10 mM lithium perchlorate as mobile phase (19). The eluate was analyzed by electrochemical detection at an applied potential of 0.6 V in an LC-4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Calibration of the HPLC system was performed daily using fresh solutions of antioxidant standards dissolved in hexane (β-carotene) or ethanol (*d,l*-α-tocopherol).

Plasma and Tissue Antioxidant Content. Tissue and plasma samples were obtained at sacrifice and stored at −70°C (protected from light) until analysis. Plasma (0.5 ml) was precipitated with an equal volume of ethanol, extracted twice with hexane, dried under nitrogen, and resuspended in mobile phase (see below) for analysis. Aortic tissue was denatured, saponified as described by Shapiro *et al.* (31), and extracted with hexane. The extract was dried under nitrogen, suspended in ethanol, pelleted, and resuspended in 25 μl of methylene chloride and 75 μl of ethanol. Sample extracts were assayed using reverse-phase HPLC with a C_{18} Baker Bond column (5 μm) using methanol/tetrahydrofuran/water/methylene chloride, 70:18:7:5 (vol/vol), with 1% ammonium acetate as a mobile phase. Retinol, α-tocopherol, and β-carotene were quantified by absorption at 325 nm, 286 nm, and 452 nm, respectively.

Pathologic Examination. Four to six animals from each group were used for pathologic examination. Thoracic aortae were isolated, cannulated with a 16-gauge stainless steel cannula, and cleared of blood with PBS (pH 7.4) for 5 min, followed by perfusion under physiologic pressure (90 mmHg) with a solution of 10% formalin in PBS for 20 min. Thoracic aortae were further fixed using cacodylate-buffered glutaraldehyde as described (32). Histologic examination was performed on 2-mm arterial segments (five sections per segment), obtained from proximal, mid, and distal portions of the aortic arch, which were embedded in paraffin and stained with resorcin fuchsin followed by morphometric analysis of intimal area and medial area using an automated video microscopy system (Image Technology, Deer Park, NY). Glutaraldehyde-fixed tissues were prepared for scanning electron microscopy as described (32) and observed in an AMR 1000-Å scanning electron microscope (Amray, Bedford, MA).

Data Analysis. All values are presented as mean ± SEM. The vascular responses to the agents acetylcholine, A23187, and sodium nitroprusside were compared among dietary groups using analysis of variance (ANOVA). Comparisons among dietary groups for lipoprotein levels, vascular contraction, antioxidant levels, and intimal-to-medial ratio were performed using ANOVA with a post hoc Neuman–Keuls comparison. Statistical significance was accepted if the null hypothesis was rejected at the $P < 0.05$ level.

RESULTS

Lipoprotein Levels. Animals fed standard chow (control group) demonstrated plasma total cholesterol, LDL-C + VLDL-C, HDL-C, and triacylglycerol levels of 57 ± 9 mg/dl, 13 ± 6 mg/dl, 30 ± 5 mg/dl, and 115 ± 15 mg/dl, respectively (Table 1). In contrast, animals receiving diets containing 1% cholesterol for 28 days demonstrated significant elevations of plasma total cholesterol, LDL-C + VLDL-C, HDL-C, and triacylglycerol levels (all $P < 0.05$ vs. control). Animals supplemented with β-carotene or α-tocopherol had plasma lipoprotein profiles similar to animals treated with 1% cholesterol alone [Table 1; P = not significant (NS)].

Dietary Antioxidants and Control of Vascular Tone. The effects of dietary β-carotene and α-tocopherol on vascular reactivity are shown in Fig. 1. Aortic rings from rabbits fed standard rabbit chow demonstrated significant dose-dependent relaxation in response to acetylcholine (Fig. 1*A*) with a maximal relaxation of 80 ± 4% ($P < 0.001$) while rabbits treated with 1% cholesterol (cholesterol group) demonstrated significantly impaired responses to acetylcholine with a maximal relaxation of 39 ± 5% ($P < 0.001$ vs. control). In contrast, rabbits fed a 1% cholesterol diet supplemented

Table 1. Plasma lipoprotein profiles of animal subjects

Plasma lipoprotein	Chow	Cholesterol	Cholesterol + β-carotene	Cholesterol + α-tocopherol
Total cholesterol, mg/dl	57 ± 9	1834 ± 226*	2042 ± 270*	1510 ± 123*
LDL-C + VLDL-C, mg/dl	13 ± 6	1494 ± 246*	1464 ± 371*	1135 ± 173*
HDL, mg/dl	30 ± 5	305 ± 74*	541 ± 194*	347 ± 92*
Triacylglycerol, mg/dl	115 ± 15	214 ± 26*	230 ± 33*	308 ± 129*

All data are expressed as the mean ± SEM and represent values taken from 9–12 animals per group. *, $P < 0.05$ compared to chow group [by analysis of variance (ANOVA) with post hoc Neuman–Keuls comparison].

with either β-carotene or α-tocopherol demonstrated maximal acetylcholine-induced vasorelaxation (Fig. 1A) that was similar to control rabbits (80 ± 6% and 83 ± 5%, respectively; P = NS vs. control).

Dietary supplementation with β-carotene and α-tocopherol produced similar effects with respect to endothelium-dependent relaxation mediated by the calcium ionophore A23187 (Fig. 1B). Vessels harvested from rabbits fed a chow diet demonstrated significant dose-dependent relaxation in response to A23187 with a maximal relaxation of 95 ± 3% ($P < 0.001$). The extent of vasorelaxation in aortic vessels from rabbits in the cholesterol group was significantly impaired relative to chow-fed animals with a maximal relaxation of 59 ± 3% ($P < 0.001$). This impairment in A23187-induced endothelium-dependent vasodilation was significantly attenuated in animals treated with β-carotene or α-tocopherol. Aortic rings harvested from these animals demonstrated dose-dependent, relaxation that was similar to that of the control group (P = NS vs. control) with maximal relaxations of 85 ± 6% (β-carotene) and 87 ± 6% (α-tocopherol).

To investigate the potential contribution of altered smooth muscle cell function to these results, we exposed study vessels to sodium nitroprusside, a direct smooth muscle relaxant and nitric oxide donor (Fig. 1C). All four groups demonstrated dose-dependent vasodilation in response to increasing concentrations of sodium nitroprusside ($P < 0.001$) with no significant differences noted on the basis of dietary treatment (Fig. 1C) or the presence of endothelium (data not shown). The contractile responses to norepinephrine were similar among the dietary treatment groups. Deendothelialized aortic rings from animals in the control, cholesterol, β-carotene, and α-tocopherol groups demonstrated similar contractions to 1 μM norepinephrine of 3.0 ± 0.8 g, 3.2 ± 1.0 g, 3.9 ± 1.4 g, and 3.4 ± 1.7 g, respectively (P = NS). Deendothelialized vessels from all four treatment groups showed no significant relaxation when exposed to A23187 and acetylcholine (data not shown) again, suggesting that changes in smooth muscle cell function do not explain the preservation of endothelium-dependent vasodilation with dietary antioxidants.

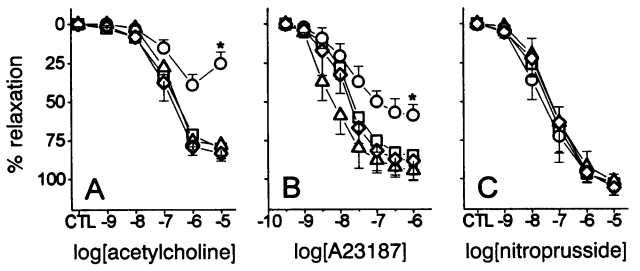

FIG. 1. Dietary antioxidants and vascular reactivity. Aortic rings from rabbits fed chow (△), 1% cholesterol (○), 1% cholesterol with β-carotene (□), and 1% cholesterol with α-tocopherol (◇) were exposed to increasing concentrations of acetylcholine (A), calcium ionophore A23187 (B), or sodium nitroprusside (C). Rabbit aortic rings (3 mm) were suspended in an organ chamber with oxygenated Krebs buffer at 37°C and contracted with 1 μM norepinephrine for the assay of vascular function as described (27). Acetylcholine, A23187, and sodium nitroprusside were added sequentially. Data are presented as the mean ± SEM in rings taken from 6–10 rabbits. CTL, control. ★, $P < 0.001$ compared to chow-fed animals (by ANOVA).

Antioxidant Levels and LDL Resistance to *ex Vivo* Oxidation. In the present study, preserved endothelium-dependent arterial relaxation may reflect β-carotene- and α-tocopherol-mediated limitation of LDL oxidation and/or atherosclerosis in these cholesterol-fed rabbits. We evaluated these possibilities by examination of vascular tissue and LDL levels of β-carotene and α-tocopherol in animals from all four treatment groups, as well as LDL susceptibility to *ex vivo* copper-mediated oxidation (Table 2). β-Carotene levels were markedly elevated in aortic tissue from β-carotene-treated animals (43.3 ± 17.3 nmol/g of tissue; $P < 0.05$ vs. control) and not detectable in the other groups. Similarly, aortic retinol (a β-carotene metabolite) levels were elevated 14-fold in β-carotene-treated animals compared to controls (3.4 ± 0.5 nmol/g of tissue vs. 0.3 ± 0.1 nmol/g of tissue; $P < 0.05$). Treatment with α-tocopherol resulted in a 10-fold increase in aortic α-tocopherol content compared with controls (63.2 ± 9.2 nmol/g vs. 6.3 ± 0.8 nmol/g; $P < 0.05$). Significant inhibition of *ex vivo* copper-mediated LDL oxidation (compared to controls) was observed only for the α-tocopherol group (183 ± 7 min vs. 135 ± 17 min; $P < 0.05$) and was associated with a 7-fold increase in LDL α-tocopherol content (Table 2). In contrast, LDL derived from β-carotene-treated rabbits contained little additional β-carotene compared to controls and demonstrated no increased resistance to copper-mediated *ex vivo* oxidation (121 ± 23 min vs. 135 ± 17 min; P = NS).

Pathologic Examination of Vascular Tissue. Previous reports have demonstrated diminished atherosclerosis in cholesterol-fed rabbits treated with the antioxidant butylated hydroxytoluene (9). To define the extent of atherosclerosis in our study animals, we performed histomorphometric analyses of thoracic aortae obtained from the four treatment groups (Fig. 2). Using the ratio of intimal area to medial area as a marker for the degree of atherosclerosis, we found no inhibition of atherogenesis with either β-carotene or α-tocopherol treatment in sections of proximal, mid, and distal aortic arch. Scanning electron microscopy of aorta in the cholesterol group demonstrated an intact endothelial layer with loss of flow alignment and occasional slight gaps between cells. Patchy areas of raised endothelial cells consistent with underlying foam-cell formation were also noted. There were no qualitative differences in endothelial cell morphology among the treatment groups fed cholesterol. It appears, therefore, that our results are not explained by antioxidant-mediated attenuation of atherogenesis or modification of endothelial cell morphology as assessed by light and scanning electron microscopy.

DISCUSSION

The data presented here demonstrate that dietary antioxidants preserve normal endothelium-dependent vasodilation in rabbits rendered hypercholesterolemic through dietary

Table 2. Tissue and LDL antioxidant levels and LDL susceptibility to *in vitro* copper-mediated oxidation in animal subjects

	Chow	Cholesterol	Cholesterol + β-carotene	Cholesterol + α-tocopherol
Aorta				
β-Carotene, nmol/g of tissue	<0.01	<0.01	43.3 ± 17.3*	<0.01
Retinol, nmol/g of tissue	0.3 ± 0.1	0.9 ± 0.2*	3.4 ± 0.5*	0.2 ± 0.1
α-Tocopherol, nmol/g of tissue	6.3 ± 0.8	16.2 ± 3.3	25.2 ± 7.1	63.2 ± 9.2*†
LDL				
β-Carotene, pmol/mg	0.7 ± 0.6	0.3 ± 0.3	5.7 ± 0.9*	2.6 ± 0.5
α-Tocopherol, nmol/mg	2.8 ± 0.5	2.7 ± 0.7	2.2 ± 0.7	19.7 ± 2.3*†
LDL oxidation lag phase, min	135 ± 17	160 ± 27	121 ± 23	183 ± 7*

All values are mean ± SEM and represent the average of six animals in each group. *, $P < 0.05$ compared to animals fed chow; †, $P < 0.05$ vs. animals fed cholesterol alone (ANOVA).

cholesterol intake. Both β-carotene and α-tocopherol, as dietary supplements, preserved EDRF-mediated control of vascular tone without any significant effects on plasma cholesterol or lipoproteins. This preservation of endothelial function was unrelated to any alteration in smooth muscle cell function as shown by the similar responses to both sodium nitroprusside and norepinephrine among dietary groups. Likewise, there was no obvious morphologic feature attributable to the normal function of the endothelium. Preservation of endothelial function was associated with increased tissue levels of lipid-soluble antioxidants and increased resistance to LDL oxidation in the α-tocopherol group but not in animals treated with β-carotene.

α-Tocopherol and β-carotene are antioxidants *in vivo* and inhibition of LDL oxidation *in vivo* may serve several important functions that may, in part, explain the preserved endothelial vasomotor function demonstrated here. Abnormalities in EDRF action induced by ox-LDL appear related to lipid peroxidation products that accumulate during the oxidation process (6, 33, 34). In particular, the production of lysolecithin during oxidative modification of LDL is central to the development of an abnormal vascular response (6, 33, 34). Incubation of ox-LDL with defatted albumin depletes the particle of lysolecithin and attenuates the development of abnormal vasomotion (6). Likewise, treatment of ox-LDL with a lysolecithinase (phospholipase B) attenuates the effect of ox-LDL on endothelium-dependent relaxation through depletion of LDL lysolecithin (33). Direct incubation of normal rabbit aortae with lysolecithin alone results in inhibition of EDRF-mediated vessel relaxation (34). Moreover, ox-LDL is toxic to endothelial cells (15) and promotes the recruitment of inflammatory cells into the vascular wall (16). Thus the limitation of LDL oxidation by α-tocopherol and β-carotene may attenuate the accumulation of lysolecithin in aortic tissue and the recruitment of inflammatory cells into developing atheroma; a situation likely to preserve endothelium-dependent vasorelaxation.

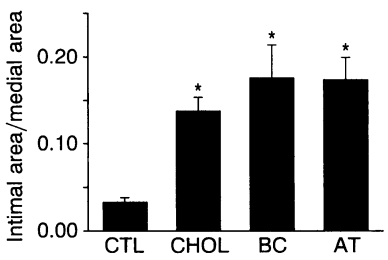

FIG. 2. Dietary antioxidants and the extent of atherosclerosis. Thoracic aortae were harvested from rabbits fed chow (CTL), 1% cholesterol (CHOL), 1% cholesterol and β-carotene (BC), or 1% cholesterol and α-tocopherol (AT). The thoracic aorta was isolated and prepared. The ratio of intimal area to medial area was used as an index of atherosclerosis. Values represent mean ± SEM of nine segments in each dietary group. ★, $P < 0.05$ vs. control by ANOVA.

Our finding that β-carotene effectively preserves endothelium-dependent relaxation without any significant effect on preventing LDL oxidation *in vitro* appears to conflict with current concepts of LDL oxidation and antioxidant protection (7). This apparent discrepancy may be related to specific properties of β-carotene. For example, β-carotene possesses radical-trapping antioxidant activity (35, 36), yet several studies have failed to demonstrate β-carotene-mediated inhibition of LDL oxidation *in vitro* (15, 26), while yet another study has demonstrated such an effect (8). The antioxidant activity of β-carotene is more efficient at low oxygen tension while α-tocopherol appears most effective at high oxygen tensions (37). It is possible, therefore, that the assay of plasma-derived LDL susceptibility to oxidation *in vitro* represents a poor assay of the antioxidant effect of β-carotene. In fact, in the present study LDL derived from β-carotene-treated rabbits contained minor amounts of β-carotene while the vascular tissue levels of β-carotene were substantial. It is likely, therefore, that any effect of β-carotene on *in vivo* LDL modification would be manifested only in the vascular wall, perhaps directly or through effects on the cell types that typically produce oxidative LDL modification. There is evidence for such an effect with the antioxidant drug, probucol (38), which appears to limit the endothelial cell capacity to modify LDL. In addition, Navab *et al.* (39) have described inhibition of cell-mediated LDL oxidation by pretreatment of endothelial cell/smooth muscle cell cocultures with α-tocopherol and β-carotene. In this same system, the addition of α-tocopherol and β-carotene concomitantly with LDL provided no protection against oxidative modification.

Abnormalities in endothelium-dependent relaxation may be related to the inflammatory response associated with atherosclerosis and hypercholesterolemia. Several cell types within atherosclerotic vessels are inflammatory in nature and capable of releasing oxygen-derived free radicals (40–43). Investigation into the chemical nature of EDRF suggests that EDRF is either nitric oxide (44) or a related redox form (45) that combines readily with a number of chemical species including oxygen (46) and superoxide anion (47) leading to a loss of biologic activity (17). One must consider that the antioxidant-mediated preservation of EDRF action described here may be a consequence of the free-radical scavenging characteristics of α-tocopherol and β-carotene vis-à-vis superoxide anion. Alternatively, these agents may in some way inhibit superoxide production by endothelial cells. Such mechanisms would reconcile the apparent discrepancy between the ability of β-carotene to preserve endothelium-dependent vasorelaxation without any protective effect on the susceptibility of LDL to copper-mediated oxidation. In fact, endothelial cells from hypercholesterolemic rabbits appear to produce excess superoxide anion (48) and enhanced degradation of superoxide anion in atherosclerotic

rabbits appears to improve the response to endothelium-dependent vasodilators (49).

Some antioxidants (vitamins A and E) may modulate some aspects of endothelial-cell function through mechanisms that are unrelated to antioxidant activity. Retinoic acid (the acid form of vitamin A) plays a role in the differentiation of several cell types including endothelial cells (50). Ishii *et al.* (51) have demonstrated retinoic acid-mediated attenuation of procoagulant activity in endothelial cells exposed to tumor necrosis factor. Melnykovych and Clowes (52) have demonstrated stimulation of endothelial cell growth by several forms of vitamin A that was particularly prominent in the presence of macrophage-conditioned medium. With regard to vitamin E, Kunasaki *et al.* (53) have observed modulation of prostaglandin synthesis in endothelial cells exposed to vitamin E while Kuzuya *et al.* (54) have demonstrated stimulation of endothelial cell proliferation in culture with exposure to *d,l*-α-tocopherol. Vitamin E enrichment of cultured endothelial cells has been shown to enhance thrombin- or A23187-stimulated arachidonic acid release (55). Thus, there is precedent for the modulation of endothelial cell function(s) by β-carotene and α-tocopherol through mechanisms unrelated to their antioxidant activity. Possible mechanisms may include increases in intracellular calcium-dependent nitric oxide synthase, alterations in intracellular L-arginine levels or transport, or modulation of signal transduction pathways that are altered in atherosclerosis.

In summary, the results presented here demonstrate that dietary treatment with the lipid-soluble antioxidants, β-carotene and α-tocopherol, preserves endothelium-dependent vasorelaxation in cholesterol-fed rabbits. These improvements in EDRF action were unrelated to any alteration in serum lipoproteins and appear independent of the extent of atherosclerosis. In particular, preservation of normal endothelium-dependent arterial relaxation was associated with the accumulation of lipid-soluble antioxidants in vascular tissue. Antioxidant-mediated preservation of normal EDRF metabolism in hypercholesterolemia may have important implications for the control of vascular tone, the prevention of local thrombosis, and the treatment of vascular disease in general. The findings presented here may represent one possible means by which antioxidant therapy may be beneficial for patients with established or developing vascular disease.

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